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Inventor: ROBERT ORROCK THOMSON

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COMPLETE SPECIFICATION

Improvements in Water-in-oil Emulsion Vaccines

We, THE WELLCOME FOUNDATION LIMITED, of 183—193 Euston Road, London, N.W.1, a company incorporated in England do hereby declare the invention for which we pray that a patent may be granted to us and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to novel vaccines comprising antigenic material in a water-in-oil emulsion.

It has been known for many years that an excellent way of enhancing antigenicity is the incorporation of the antigenic material in a water-in-oil emulsion. The best-known water-in-oil vaccines make use of Freund adjuvant, which comprises paraffin oil (Bayol F or Drakeol No. 6 R) together with an anhydrohexitol monooleate (Arlacel A) as emulsifier, and also killed mycobacteria in the complete Freund adjuvant. However, other oils and emulsifiers are used. A typical water-in-oil vaccine, as has been used for example in clinical trials of influenza vaccine, comprises an aqueous antigen preparation blended with an equal volume of paraffin oil (for example, Drakeol No. 6 R) itself containing 10% emulsifier (Arlacel A) (Trade Mark).

In spite of their very high antigenicity, however, water-in-oil vaccines have not been widely used because undesirable tissue reactions occur at the site of injection. These reactions have been ascribed both to the hydrocarbon oil and to the emulsifier, and as far as the latter is concerned it appears that emulsifiers chosen to give little or no reaction also give very poorly antigenic vaccines.

It has now been discovered that the concentration both of the aqueous phase and of the emulsifier in a water-in-oil vaccine can be substantially reduced, and the amount of oil correspondingly increased, affording a substantial reduction in the incidence and extent of undesirable reaction at the site of injection, but without diminishing the antigenic efficiency of a given amount of antigenic material. The viscosity of the vaccine is also reduced, compared with the undesirably high viscosity of conventional water-in-oil vaccines.

According to the invention, in a vaccine comprising antigenic material in a water-in-oil emulsion, the aqueous phase constitutes less than 20% by volume of the total volume of the vaccine, and the emulsifier constitutes less than half but at least one-tenth by volume of the volume of the aqueous phase. Preferably, the aqueous phase constitutes from 1% to 10% of the total volume of the vaccine, and the volume of the emulsifier is one-third of the volume the aqueous phase.

Vaccines according to the invention may be produced in similar fashion to conventional water-in-oil vaccines by blending an aqueous antigen preparation with a dispersion of emulsifier in oil, employing constituents in the novel proportions. Alternatively, a water-in-oil vaccine of the conventional type can be diluted with oil if its content of antigenic material remains sufficient on dilution to ensure adequate immunity. It is preferable to add the aqueous phase slowly to the oil phase with agitation in order to minimise any tendency to form an oil-in-water emulsion. Finer dispersion of the aqueous phase can be obtained by homogenising or milling in a collodial mill, and this may be continued until no further decrease in particle size occurs. Inversion of the emulsion to an oil-in-water emulsion or breaking of the emulsion may take place if the agitation is prolonged or too violent.

The antigenic material for incorporation in the vaccine may be any antigen or mixture of antigens desired for immunising vertebrates; for example it may be of bacterial, viral or fungal origin or derived from protozoal or metazoal parasites or it may be a preparation of pollen or other allergens. The preparation of suitable antigenic material is fully described in the literature and particularly in

such monographs as the *British Pharmacopoeia, Pharmacopoeia* of the United States of America, *British Veterinary Codex* and the *Dispensatory of the United States of America.*

The invention has found most useful application to vaccines used to combat histotoxic clostridial diseases which attack adult cattle, sheep, lambs in many parts of the world. The antigenic material may therefore be derived from one or more bacteria which are causative organisms of these diseases and include *Clostridium perfringens* Type B, *Cl. perfringens* Type C, *Cl. perfringens* Type D, *Cl. chauvoei, Cl. septicum, Cl. oedematiens* and *Cl. tetani*. Antigenic material obtained from these organisms is often combined to give multi-component vaccines for treating more than one disease, and the invention may be applied also to these multi-component vaccines, for example a vaccine for treating Braxy and Blackleg containing antigenic material derived from *Cl. septicum* and *Cl. chauvoei*, a vaccine for treating Braxy, Blackleg and Black Disease derived from *Cl. septicum, Cl. chauvoei* and *Cl oedematiens*, and a vaccine for treating Braxy, Blackleg, Pulpy Kidney and tetanus diseases derived from *Cl. septicum, Cl. chauvoie, Cl. perfringens* Type D, and *Cl. tetani*. The invention may also be used in the preparation of *Br. abortus* vaccines such as a killed *Br. abortus* 45/20 strain vaccine.

Naturally, the emulsifiers for inclusion in vaccines of the invention must be non-toxic and compatible with the antigenic components of the vaccine. Lipophilic emulsifiers are most suitable and may be non-ionic surface active agents which have a low hydrophile-lipophile balance of about between 8 and 2. [William G. Griffin, "Calculation of HLB values of non-ionic surfactants", *Journal of the Society of Cosmetic Chemists* (1954) volume 5, pages 249—256; Osipow, "*Surface Chemistry, ACS Monograph 153*", pages 295—314, Reinhold, 1962]. This is the outer limit of the range of HLB values of emulsifiers suitable and a preferred limit of HLB values is 2 to 6 with an optimum range of 3 to 5. Among suitable lipophilic emulsifiers are di- and tri- esters of polyhydric alcohols and fatty acids, oxidised fatty oils, and partial esters of common fatty acids, e.g. palmitic, lauric, stearic and oleic acids with hexitol anhydrides derived from sorbitol or mannitol. Examples of specific emulsifiers are mannide monooleate (Arlacel A; HLB 4.3), sorbitan monooleate (Arlacel 80; HLB 43), sorbitan monostearate (Arlacel 60; HLB 4.7), and sorbitan sesquioleate (Arlacel C, HLB 3.7).

Any non-toxic oil of vegetable or mineral origin compatible with the antigenic material may be used in the vaccines of this invention and may be for example, paraffin oil (Bayol F [Trade Mark] or Drakeol No. 6 R), cottonseed oil, peanut oil, light white mineral oil (Marcol GX) (Trade Mark), or mineral seal oil.

The following examples illustrate the invention.

EXAMPLE 1 — Pulpy Kidney vaccine.

A toxigenic strain of *Clostridium perfringens* Type D was grown in a liquid medium under conditions to ensure adequate production of epsilon toxin and/or prototoxin. The culture was treated with formaldehyde to convert the epsilon toxin and prototoxin into epsilon toxoid and render the product non-toxic to mice. The bacteria were removed and the filtrate was then diluted to give four aqueous antigen preparations whose concentrations of *Clostridium perfringens* epsilon toxoid were in the ratio 1:3:9:27. These preparations satisfied the requirements of the *British Veterinary Codex* with regard to safety, sterility and freedom from abnormal toxicity.

Each of the aqueous antigen preparations (30 parts) was made into a water-in-oil emulsion of the conventional type by blending with a mixture of "Bayol F" mineral oil (59.5 parts) and "Arlacel A" mannide monooleate (10.5 parts). The three more concentrated emulsions, whose antigen contents were in the ratio 3:9:27, were then diluted 3-fold, 9-fold and 27-fold respectively with "Bayol F" mineral oil to give vaccines according to the present invention, all having the same antigen content but having different proportions of aqueous phase, oil and emulsifier. These vaccines were much superior to the conventional emulsion with regard to tissue reaction at the site of injection; this reaction decreased with increasing dilution of the aqueous phase and emulsifier with oil. Their antigenic potency, on the other hand, remained unimpaired. This was illustrated by antigenicity tests in two groups of 10 mice, each of which received two 0.5 ml subcutaneous injections 4 weeks apart of one emulsion, the circulating antibody being measured 14 days later after pooling the blood from each group. The three diluted emulsions were thus compared with the least concentrated undiluted conventional emulsion. The results are given in the following table, in which the titre is at least as great as the value given but less than twice that value.

Composition of pulpy kidney vaccine			Antibody titre (u/ml)	
Bayol F	Arlacel A	Aqueous phase	Group 1	Group 2
59.5%	10.5%	30.0%	10	20
86.5%	3.5%	10.0%	20	20
95.5%	1.2%	3.3%	20	40
98.5%	0.4%	1.1%	10	10

EXAMPLE 2 — Black disease vaccine

A toxigenic strain of *Clostridium oedematiens* Type B was grown in a liquid medium. The culture was then treated with formaldehyde until the product was sterile and non-toxic. The bacteria were removed by filtration and the filtrate was concentrated by precipitation with ammonium sulphate, dispersing the precipitate in water and dialysing to remove ammonium sulphate. The resultant concentrate was then diluted to give three aqueous antigen preparations whose concentrations of *Clostridium oedematiens* alpha toxoid were in the ratio 1:3:9. These preparations satisfied the requirements of the *British Veterinary Codex* with regard to safety, sterility and freedom from abnormal toxicity.

The most dilute aqueous antigen preparation (30 parts) was made into a water-in-oil emulsion of the conventional type of blending with a mixture of "Bayol F" mineral oil (59.5 parts) and "Arlacel A" mannide monooleate (10.5 parts), and was then diluted 3-fold with "Bayol F" mineral oil to give a vaccine according to the present invention. Other vaccines according to the invention, all having the same antigen content but having different proportions of aqueous phase, oil and emulsifier, were prepared by emulsifying the three aqueous antigen preparations (whose antigen contents were in the ratio 1:3:9) by blending 10 parts, 3.3 parts, and 1.1 parts respectively with mixtures of "Bayol F" mineral oil (86.5 parts, 95.5 parts, and 98.5 parts respectively) and "Arlacel A" mannide monooleate (3.5 parts, 1.2 parts, and 0.4 part respectively). These vaccines were much superior to a conventional emulsion with regard to tissue reaction at the site of injection; this reaction decreased with increasing dilution of the aqueous phase and emulsifier with oil. Their antigenic potency, on the other hand, remained unimpaired. For example, in the following table are given the results of antigenicity tests in groups of 10 mice, each of which received two 0.5 ml subcutaneous injections 4 weeks apart of one of the emulsions (all of which had the same antigen content), the circulating antibody being measured 14 days later after pooling the blood from each group; in these results the titre is at least as great as the value given but less than twice that value.

Composition of Black disease vaccine			Antibody titre (u/ml)	
Bayol F	Arlacel A	Aqueous phase	Group 1	Group 2
86.5%	3.5%	10.0% (a)	100	40
86.5%	3.5%	10.0% (b)	40	40
95.5%	1.2%	3.3% (b)	40	20
98.5%	0.4%	1.1% (b)	20	20

(a) made by diluting a conventional emulsion with oil.

(b) made by emulsification of the ingredients directly.

EXAMPLE 3 — Mutiple clostridial vaccine

A toxigenic strain of *Clostridium perfringens* Type B was grown in a liquid medium to ensure adequate production of beta and epsilon toxins and/or protoxin. The culture was treated with formaldehyde until the product was sterile and non-toxic to mice. The bacteria were removed to give an aqueous preparation containing *Clostridium perfringens* beta and epsilon toxoids (lamb dysentry vaccine).

A toxigenic strain of *Clostridium perfringens* Type C was grown in a liquid medium to ensure adequate production of beta toxin. The culture was treated with formaldehyde until the product was sterile and non-toxic to mice. The bacteria were removed to give an aqueous preparation containing *Clostridium perfringens* beta toxoid (Struck vaccine).

A sterile filtrate from a culture of *Clostridium tetani* containing tetanus toxin was treated with formaldehyde at 37°C for several weeks and then purified by ultrafiltration and precipitation with potassium phosphate to give an aqueous preparation of tetanus toxoid satisfying the requirements of the *British Pharmcopoeia*.

A toxigenic strain of *Clostridium septicum* was grown in a liquid medium under conditions to ensure adequate production of toxin. The culture was then treated with formaldehyde until the product was sterile and non-toxic to mice. The bacteria were removed to give an aqueous preparation of *Clostridium septicum* toxoid (braxy vaccine).

A toxigenic strain of *Clostridium chauvoei* was grown in a liquid medium under conditions to ensure adequate production of toxin. The culture was then treated with formaldehyde until the product was sterile and non-toxic to mice. The bacteria were removed to give an aqueous proparation containing *Clostridium chauvoei* toxoid (blackleg vaccine).

Aqueous preparations containing the beta and epsilon toxoids of *Clostridium perfringens* (from Types B, C and D) and the toxoids of *Clostridium septicum* and *Clostridium oedematiens*, each obtained as described above or in Examples 1 and 2 and concentrated as described in Example 2, were mixed together with aqueous preparations of tetanus toxoid and *Clostridium chauvoei* toxoid obtained as described above. This mixture as the aqueous phase (30 parts) was blended with a mixture of "Bayol F" mineral oil (56 parts), "Arlacel A" mannide monooleate (10.5 parts) and "Falba" absorption base (3.5 parts), or with a mixture of "Bayol F" mineral oil (59.5 parts) and "Arlacel A" mannide monooleate (10.5 parts). ("Falba" is an emulsion stabiliser which contains beeswax, paraffin oils of varying viscosities, and oxycholesterine extracted from lanolin.) The resultant emulsions were then diluted 4-fold with "Bayol F" mineral oil to give vaccines according to the invention.

These vaccines were tested for antigenic potency in rabbits, guinea pigs and sheep and were found to give protection against all the seven diseases they were designed for (lamb dysentery, struck, pulpy kidney, tetanus, braxy, blackleg and black disease) in excess of the requirements of the *British Veterinary Codex*. Their stability was satisfactory in tests at 2°C and 37°C. Both viscosity and tissue reaction were less than for the undiluted emulsion.

EXAMPLE 4

To a suspension (16 ml.) of ethanol killed *Brucella abortus* 45/20 stratin in distilled water having an opacity of 250 International Units, was added Arlacel A (4 ml.) and the resulting suspension emulsified with 100 ml. Bayol 55. Before use, the emulsion produced was diluted with a further volume of Bayol 55 (80 ml.).

This vaccine was tested for antigenic potency in guinea pigs and was found to give protection against challenge by a virulent strain *Brucella abortus* No. 544. Seven out of the nine guinea pigs were subsequently found to have no *Brucella abortus* in their spleens. An uninoculated control group challenged with the same virulent strain showed heavy colonisation of all the spleens with a mean of 450×10^3 organisms per spleen.

The stability of the vaccine was satisfactory at tests at 2°C and 37°C. Both viscosity and tissue reaction were less than an undiluted emulsion of the same vaccine.

WHAT WE CLAIM IS:—

1. A water-in-oil emulsion vaccine comprising an oil phase, an emulsifier, and an aqueous phase containing antigen, wherein the aqueous phase constitutes less than 20% by volume of the total volume of the vaccine, and the emulsifier constitutes less than one-half but at least one-tenth by volume of the volume of the aqueous phase.

2. A vaccine as claimed in claim 1 wherein the aqueous phase constitutes from 1% to 10% by volume of the total volume of the vaccine.

3. A vaccine as claimed in claim 1 or 2 wherein the volume of the emulsifier is one-third of that of the aqueous phase.

4. A vaccine as claimed in any preceding claim wherein the emulsifier is a non-ionic surface active agent.

5. A vaccine as claimed in claim 4 wherein the emulsifier has an H.L.B. value of from 8 to 2 inclusive.

6. A vaccine as claimed in claim 5 wherein the emulsifier has an H.L.B. value of from 3 to 5 inclusive.

7. A vaccine as claimed in claim 6 wherein the emulsifier is mannide monooleate.

8. A vaccine as claimed in any preceding claim wherein the antigen is derived from a non-virulent strain of *Brucella abortus*.

9. A vaccine as claimed in claim 8 wherein the antigen comprises killed microorganisms of *Brucella abortus strain* 45/20.

10. A vaccine as claimed in any of claims 1 to 7 wherein the antigen comprises an antigen derived from microorganisms of the genus *clostridium*.

11. A vaccine as claimed in any preceding claim wherein the oil is a mineral oil.

12. A vaccine as claimed in any preceding claim wherein the oil is paraffin oil.

13. A vaccine substantially as hereinbefore described in any one of Examples 1 to 4.

R. F. HASLAM,
Agent for the Applicants.
Chartered Patent Agent.

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